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Comparison of the effects of crocin, safranal and diclofenac on local inflammation and inflammatory pain responses induced by carrageenan in rats

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Abstract:
Background: Crocin and safranal are the active substances of saffron and have many biological properties. In the present study, we compared the effects of crocin, safranal and diclofenac on local inflammation and its induced pain in rats.
Methods: Local inflammation was induced by intraplantar (*ipl*) injection of carrageenan (100 µl, 2%). Paw thickness was measured before and after carrageenan injection. Inflammatory pain responses including cold allodynia, mechanical allodynia and hyperalgesia were assessed using acetone spray and von Frey filament tests, respectively. The number of neutrophils in inflammatory zone was counted 6.5 h after injection of carrageenan.
Results: Carrageenan produced edema, cold allodynia, mechanical allodynia and hyperalgesia and caused neutrophil infiltration in paw tissues. Crocin at doses of 25, 50 and 100 mg/kg, safranal at doses of 0.5, 1 and 2 mg/kg and diclofenac (as a reference drug) at a dose of 10 mg/kg attenuated edema, suppressed inflammatory pain responses and decreased the number of neutrophils.
Conclusion: The present study showed anti-inflammatory and antinociceptive activities for crocin, safranal and diclofenac in carrageenan model of local inflammation and inflammatory pain.

Key words: crocin, safranal, diclofenac, edema, inflammatory pain, rats

Introduction

It has been accepted that all pain, whether acute or chronic, peripheral or central, originates from inflammation and the inflammatory responses [29]. It is well known that tissue damage is associated with the release of several inflammatory mediators such as hydrogen ions, adenosine triphosphate, glutamate, bradykinin, cytokines, histamine, serotonin and prostaglandins that cause sensitization or activation of peripheral nociceptors [20]. The sensitization of primary afferent nociceptors is a common denominator of all kinds of inflammatory pain that leads to a state of hyperalgesia and/or allodynia, better described as hypernociception in animal models [25, 36]. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used drugs in inflammatory diseases, since they are effective in management of pain, fever,

redness and edema resulting from inflammatory mediator release [39]. Due to the significant side effect profiles of NSAID medications, there is a great interest in natural compounds, such as dietary supplements and herbal remedies, which have been used for centuries to reduce pain and inflammation [23].

Crocus sativus L., commonly known as saffron, is used in folk medicine for various purposes such as: antispasmodic, nerve sedative, expectorant, eupeptic, anticatarrhal, carminative, diaphoteric, stomachic, aphrodisiac and emmenagogue [37]. Saffron contains carotenoid pigments called tricrocin, bicrocin and crocin, a bitter glycoside called picocrocin, and the volatile, aromatic substance safranal [32]. Pharmacological studies have demonstrated antiepileptic, neuroprotective, anti-diabetic, antioxidant, anti-inflammatory and antinociceptive properties for crocin and safranal [2, 27, 40–45]. Xu et al. [50] found that oral administration of crocin decreased paw edema induced by carrageenan in rats. In the formalin, writhing and hot plate tests of nociception, safranal showed antinociceptive and anti-inflammatory properties [17]. The antinociceptive effects of crocin have been shown in corneal nociception and formalin test in rats [43, 44]. More recently, Amin and Hosseinzadeh, [1] showed that safranal, but not crocin, attenuated pain responses in chronic constriction injury model of neuropathic pain in rats.

In the present study, we compared the effects of crocin, safranal and diclofenac on edema, allodynia and hyperalgesia induced by intraplantar (*ipl*) injection of carrageenan in rats. The *ipl* injection of carrageenan is a common model to study inflammation and inflammatory pain. Carrageenan produces edema and causes an exacerbated sensitivity to thermal and mechanical stimuli that is known as hyperalgesia [14, 28, 31, 33]. The commonly prescribed NSAID, diclofenac, has been shown to inhibit the inflammation and hypersensitivity induced by carrageenan in rats [10, 14], and was therefore included in this study as a reference drug.

Materials and Methods

Animals

Healthy adult male Wistar rats, weighing 230–260 g were used in this study. The animals were provided from rat house of Laboratory of Physiology of Faculty of Veterinary Medicine of Urmia University. Rats were maintained in groups of six per cage in a 12 h light-dark cycle (light on at 7:00 h) at a controlled ambient temperature (22 ± 0.5°C) with *ad libitum* food and water. Six rats were used for each experiment. All experiments were performed between 12:00 h and 19:00 h. All research and animal care procedures were approved by the Veterinary Ethics Committee of the Faculty of Veterinary Medicine of Urmia University (Ref. No. AECVU/127/2012), and were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Drugs

Drugs used in the present study included crocin, safranal, λ-carrageenan and diclofenac sodium (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Crocin, diclofenac and λ-carrageenan were dissolved in normal saline. Safranal was dissolved in liquid paraffin [1].

Treatment protocol

The animals were divided into following 12 groups of six rats each:

Group 1: This group received *ip* injection of normal saline or paraffin followed by *ipl* injection of normal saline.

Group 2: This group received *ip* injection of normal saline followed by *ipl* injection of carrageenan, and served as a control group for crocin treated groups.

Groups 3, 4, 5 and 6: These groups received *ip* injection of crocin at doses of 12.5, 25, 50 and 100 mg/kg, respectively, followed by *ipl* injection of carrageenan.

Group 7: This group received *ip* injection of paraffin followed by *ipl* injection of carrageenan, and served as a control group for safranal treated groups.

Groups 8, 9, 10 and 11: These groups were treated with *ip* injection of safranal at doses of 0.25, 0.5, 1 and 2 mg/kg, respectively, before *ipl* injection of carrageenan.

Group 12: In this group, *ipl* injection of carrageenan was performed after *ip* injection of diclofenac at a dose of 10 mg/kg.

The *ip* injections of crocin, safranal and diclofenac were performed 30 min before *ipl* injections of carrageenan. In the present study, the doses of crocin, safranal and diclofenac were designed according to

previous studies, which were at 50–200 mg/kg, 0.025–1 mg/kg and 2.5-10 mg/kg, respectively [5, 14, 15, 43, 44].

Local edema induction

For induction of local edema, each rat was injected (*sc*) with 100 μl of λ-carrageenan 2% in the ventral surface of the right hind paw using a 26-gauge injection needle. The magnitude of paw edema was assessed by measuring the dorsal-plantar paw thickness with a fine caliper at 1 h before and 1, 2, 3, 4, 5 and 6 h after carrageenan injection. Edema was expressed as the increase in paw thickness (mm) after carrageenan injection relative to the pre-injection value for each animal [14].

Nociceptive tests

Cold allodynia was measured using acetone spray test as described by Tegeder et al. [46]. Absolute acetone was gently applied to the mid-plantar surface of the rat with a syringe connected to a thin polyethylene tube. The time rat spent licking and lifting the acetone-sprayed paw was recorded with a stopwatch for a period of 3 min starting right after acetone application. Cold allodynia was recorded 1 h before and 1, 3 and 5 h after carrageenan injection. Mechanical allodynia and hyperalgesia were assessed using an electronic von Frey Anesthesimeter (IITC-Life Science Instruments, Woodland Hill, CA, USA) as described by Chaplan et al. [8] and Choi et al. [9]. Briefly, the rats were placed in individual Plexiglas chambers (18 × 10 × 20 cm) with wire mesh floor, and allowed to explore and groom until they settled down. Two von Frey filaments with bending forces of 2.5 and 28 g (No. 8 and 14) were applied to the plantar surface of the right hind paw. Hind paw withdrawal was considered as positive response. The stimulation with filaments was repeated 10 times at 10–15 s intervals. Mechanical allodynia was recorded 1.5 h before and 1.5, 3.5 and 5.5 h after carrageenan injection. Mechanical hyperalgesia was recorded 2 h before and 2, 4 and 6 h after injection of carrageenan. The response frequency to von Frey filament application was expressed as percent of response frequency ([number of paw withdrawals/number of trails] × 100) [51]. Bravo-Hernandez et al. [6] have been reported that the occurrence of responses in normal rats to the low and high forces indicates mechanical allodynia and mechanical hyperalgesia, respectively. Mechanical allodynia and hyperalgesia were considered as primary as stimulation with von Frey filaments were applied to the carrageenan injected sites.

Histopathological evaluation

The animals were euthanized by decapitation 6.5 h after carrageenan injection, and their paw tissues were collected for histopathological investigation. The specimens were fixed in 10% buffer formal saline and were routinely processed for paraffin embedding. For each sample, 4 μm thick sections were cut and stained with hematoxylin-eosin, to evaluate the acute inflammation. Neutrophils were counted by special morphometric lens in 0.25 mm^2 microscopic field, from 10 different areas of the sections and the mean values were calculated. The final number of neutrophils was expressed as the mean of the number counted in six animals per group.

Statistical analysis

Statistical analysis was performed by factorial analysis of variance (ANOVA) and Duncan's test for the data obtained from the paw edema, cold allodynia, mechanical allodynia and mechanical hyperalgesia. Data obtained from the number of neutrophils were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test. The significance level was expressed as $p < 0.05$.

Results

The results of paw thickness, cold allodynia, mechanical allodynia and mechanicl hyperalgesia obtained from the hours before and after *ipl* injection of normal saline did not show any significant differences. Therefore, the results belong to the hours after *ipl* injection of normal saline have not been shown in figures.

Figure 1 shows the local edema induced by *ipl* injection of carrageenan and the effects of *ip* injections of normal saline or paraffin, crocin, safranal and diclofenac on carrageenan-induced edema. The local edema induced by carrageenan showed a significant difference between 1 and 2 h with 3, 4, 5 and 6 h after carrageenan injection (Figs. 1A, 1B). The edema responses induced by carrageenan had the same charac-

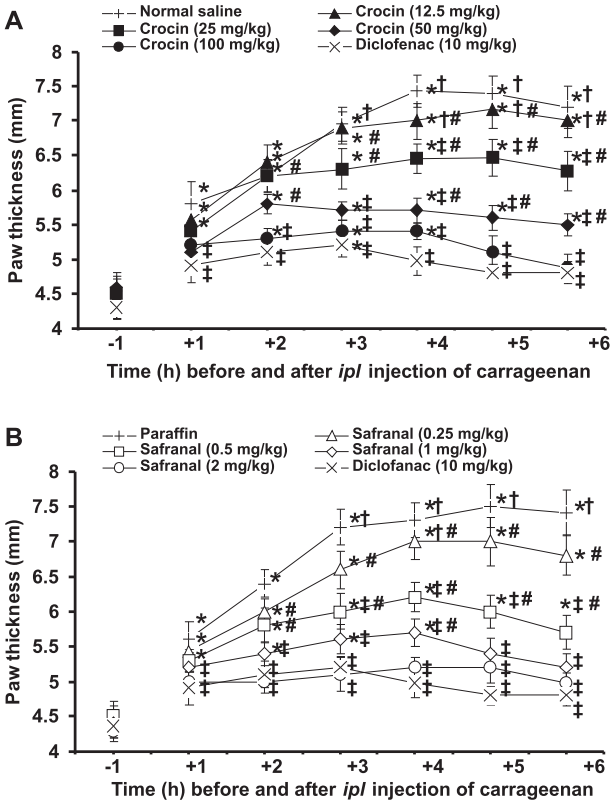

Fig. 1. Effects of crocin (**A**), safranal (**B**) and diclofenac on carrageenan-induced paw edema. Crocin, safranal and diclofenac were intraperitoneally injected 30, 35 and 25 min, respectively, before intraplantar (*ipl*) injection of carrageenan. Values are expressed as the mean ± SEM (n = 6). * $p < 0.05$ compared with -1 h, † $p < 0.05$ compared with +1 h and +2 h, ‡ $p < 0.05$ compared with normal saline, # $p < 0.05$ compared with diclofenac (10 mg/kg)

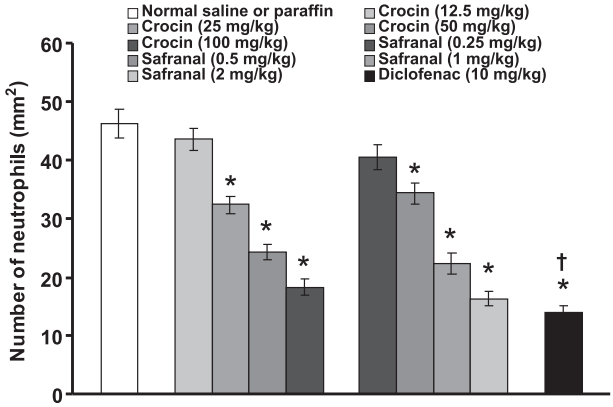

Fig. 2. Effects of crocin, safranal and diclofenac on the number of infiltrated neutrophils induced by carrageenan in paw tissues. Crocin, safranal and diclofenac were intraperitoneally injected 30, 35 and 25 min, respectively, before intraplantar (*ipl*) injection of carrageenan. Values are expressed as the mean ± SEM (n = 6). * $p < 0.05$ compared with normal saline; † $p < 0.05$ compared with crocin (12.5, 25 and 50 mg/kg) and safranal (0.25, 0.5 and 1 mg/kg)

ters in both normal saline and paraffin treated groups (Figs. 1A, 1B). Crocin (12.5 mg/kg) and safranal (0.25 mg/kg) produced no significant effects, whereas 25, 50 and 100 mg/kg of crocin [$F(6, 254) = 71.012$, $p < 0.05$, Fig. 1A] and 0.5, 1 and 2 mg/kg of safranal [$F(6, 254) = 54.501$, $p < 0.05$, Fig. 1B] significantly reduced carrageenan-induced paw edema. Diclofenac (10 mg/kg) significantly ($p < 0.05$) decreased the edema induced by carrageenan (Fig. 1A, 1B). No significant differences were observed among the suppressive effects of 100 mg/kg of crocin, 2 mg/kg of safranal and 10 mg/kg of diclofenac on carrageenan-induced edema (Fig. 1A, 1B).

Figures 2 and 3 show the neutrophil infiltration induced by *ipl* injection of carrageenan in paw tissues and the effects of *ip* injections of crocin, safranal and diclofenac on infiltration of neutrophils. Normal saline or paraffin did not induce any neutrophil in paw

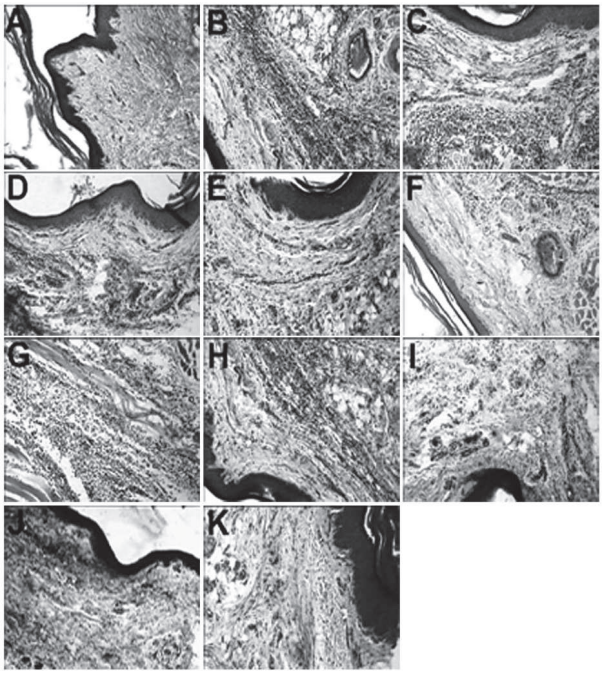

Fig. 3. Effects of intraperitoneal (*ip*) injections of crocin, safranal and diclofenac on neutrophil infiltration induced by intraplantar injection of carrageenan in rat paw tissues. Animals were treated with: (**A**) normal saline or paraffin + normal saline and (**B**) normal saline or paraffin; (**C**) crocin (12.5 mg/kg); (**D**) crocin (25 mg/kg); (**E**) crocin (50 mg/kg); (**F**) crocin (100 mg/kg); (**G**) safranal (0.25 mg/kg); (**H**) safranal (0.5 mg/kg); (**I**) safranal (1 mg/kg); (**J**) safranal (2 mg/kg) and (**K**) diclofenac (10 mg/kg), before intraplantar (*ipl*) injection of diclofenac. No infiltration is seen in **A**. Extensive infiltration of neutrophils is seen (**B**, **D** and **H**). Moderate neutrophilic infiltration observed (**E**, **F**, **I** and **J**). Mild infiltration of neutrophils (**C**, **G** and **K**) is seen (H&E, 100×)

tissues (Fig. 3A). As presented in Figure 2 and showed in Figure 3B, the number of neutrophils showed its peak (46.2 ± 2.4) in carrageenan-injected group. The infiltration of neutrophils was not influenced by 12.5 mg/kg of crocin and 0.25 mg/kg of safranal (Figs. 2, 3C and 3G). Crocin at doses of 25, 50 and 100 mg/kg [$F(5, 30) = 63.769$, $p < 0.05$ (Figs. 2, 3D, 3E and 3F)] and safranal at doses of 0.5, 1 and 2 mg/kg [$F(5, 30) = 52.078$, $p < 0.05$ (Figs. 2, 3H, 3I and 3J)] significantly decreased the number of neutrophils. Diclofenac (10 mg/kg) significantly ($p < 0.05$) suppressed the number of neutrophils in paw tissues (Figs. 2 and 3K). No significant differences were observed among the neutrophil reducing effects of 100 mg/kg of crocin, 2 mg/kg of safranal and 10 mg/kg of diclofenac (Figs. 2, 3F, 3J and 3K).

Figures 4 and 5 show the inflammatory pain responses including cold allodynia, mechanical allodynia and hyperalgesia induced by *ipl* injection of carrageenan and the effects of *ip* injections of crocin, safranal and diclofenac on inflammatory pain responses. The same inflammatory responses were induced by carrageenan in both normal saline and paraffin treated groups (Figs. 4 and 5). Crocin at a dose of 12.5 mg/kg and safranal at a dose of 0.25 mg/kg produced no significant effects on inflammatory pain (Figs. 4 and 5). Crocin at doses of 25, 50 and 100 mg/kg significantly reduced cold allodynia [$F(6, 140) = 66.978$, $p < 0.05$ (Fig. 4A)], mechanical allodynia [$F(6, 140) = 24.682$, $p < 0.05$, (Fig. 4B)] and mechanical hyperalgesia [$F(6, 140) = 26.517$, $p < 0.05$ (Fig. 4C)]. Safranal at doses of 0.5, 1 and 2 mg/kg also significantly reduced cold allodynia [$F(6, 140) = 74.868$, $p < 0.05$ (Fig. 5A)], mechanical allodynia [$F(6, 140) = 18.568$, $p < 0.05$ (Fig. 5B)] and mechanical hyperalgesia [$F(6, 140) = 33.056$, $p < 0.05$ (Fig. 5C)]. Diclofenac (10 mg/kg) significantly ($p < 0.05$) suppressed inflammatory pain induced by carrageenan (Figs. 4 and 5). No significant differences were observed among 100 mg/kg of crocin, 2 mg/kg of safranal and 10 mg/kg of diclofenac in reducing the allodynia and hyperalgesia (Figs. 4 and 5).

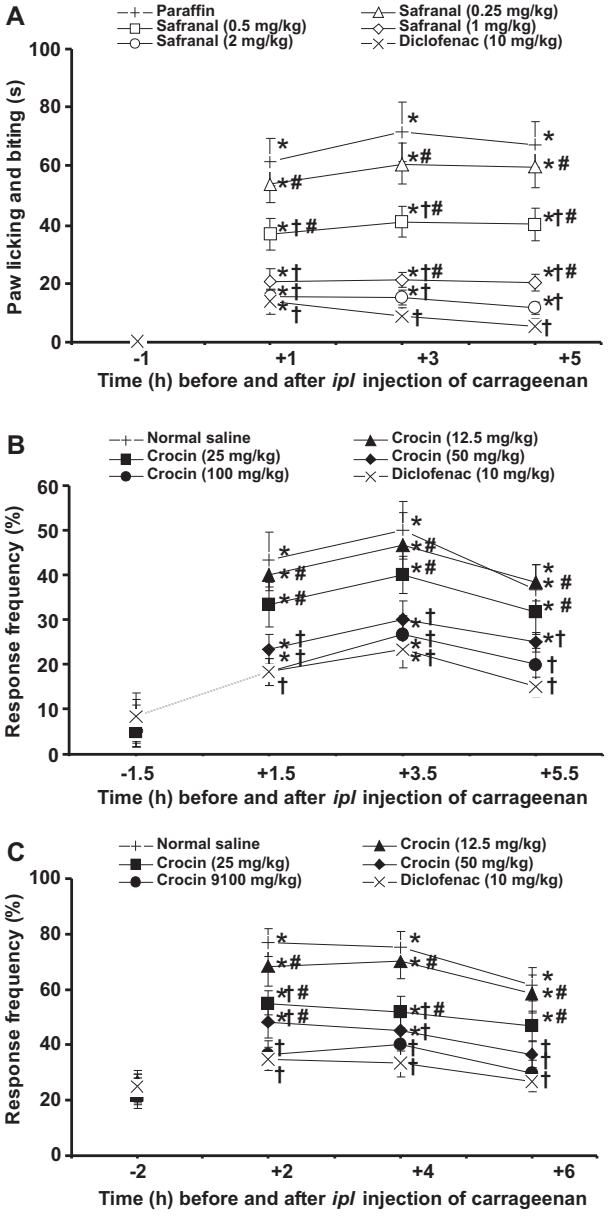

Fig. 4. Effects of crocin and diclofenac on cold allodynia (**A**), mechanical allodynia (**B**) and mechanical hyperalgesia (**C**) induced by carrageenan. Crocin and diclofenac were intraperitoneally injected 30 and 25 min, respectively, before intraplantar (*ipl*) injection of carrageenan. Values are expressed as the mean ± SEM (n = 6). * $p < 0.05$ compared with -1 h, † $p < 0.05$ compared with normal saline, # $p < 0.05$ compared with diclofenac (10 mg/kg)

Discussion

In this study, *ipl* injection of carrageenan (100 µl, 2%) produced a local edema initiated 1 h after injection, reached its maximum rate at 3 h post injection and continued to the end of the experiment. Carrageenan also caused neutrophil infiltration in paw tissues and produced cold allodynia, mechanical allodynia and hyperalgesia. Diclofenac attenuated edema and subsequent inflammatory pain. It has been reported that carrageenan produced a biphasic pattern (an early

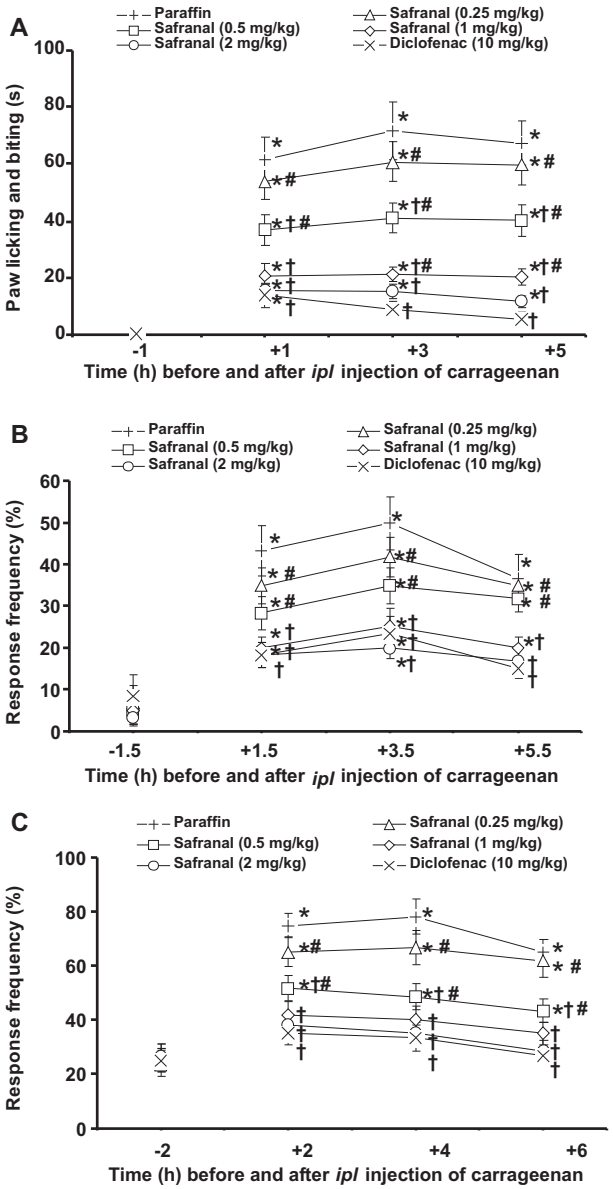

Fig. 5. Effects of safranal and diclofenac on cold allodynia (**A**), mechanical allodynia (**B**) and mechanical hyperalgesia (**C**) induced by carrageenan. Safranal and diclofenac were intraperitoneally injected 35 and 25 min, respectively, before intraplantar (*ipl*) injection of carrageenan. Values are expressed as the mean ± SEM (n = 6). * $p < 0.05$ compared with –1 h, † $p < 0.05$ compared with normal saline, # $p < 0.05$ compared with diclofenac (10 mg/kg)

phase: up to 1 h to 2 h and a late phase up to 2 h to 6 h) of paw edema in rats [14, 21, 38]. Some researchers found that 3 h and 4 h were the beginning of the late phase of paw edema after *ipl* injection of carrageenan [24, 34, 35]. Paw inflammation induced by carrageenan is a useful model to assess the contribution of inflammatory mediators involved in vascular changes and neutrophilic infiltration associated with an acute inflammatory response [26]. The early phase of carrageenan-induced paw edema is attributed to a release of inflammatory mediators such as histamine and serotonin and late phase is due to a release of pro-inflammatory mediators such as prostaglandin-like substances [21]. It has been reported that infiltrated neutrophils into the inflamed area participate in the cascade of events leading to mechanical hypernociception [11]. The inflammatory pain responses induced by carrageenan observed in the present study, are in agreement with other findings in which cold and mechanical hypersensitivity have been reported after *ipl* injection of carrageenan in rats [7, 22, 46]. It is known that diclofenac, as other NSAIDs, is able to impair prostaglandin synthesis by the inhibition of COX-1 and COX-2 pathways in both, the injured tissue and the central nervous system [49]. In addition, diclofenac reduced the number of neutrophils in carrageenan-induced paw inflammation in rats [14]. It has been reported that infiltrated neutrophils-released prostaglandin E_2 directly sensitizes mechanical nociceptors to produce hypernociception [12]. Prostaglandin E_2 has an important role in producing inflammatory hyperalgesia [19].

The present results showed that crocin and safranal suppressed paw edema induced by *ipl* injection of carrageenan. The suppressive effects of crocin and safranal on paw edema were dose-dependent because low doses of crocin (12.5 mg/kg) and safranal (0.25 mg/kg) produced no effect, whereas crocin (25 mg/kg) and safranal (0.5 mg/kg) suppressed the late phase of carrageenan-induced paw edema. By increasing the dose of crocin to 50 and 100 mg/kg and safranal to 1 and 2 mg/kg, both phases of paw edema were suppressed. Xu et al. [50] found that oral administration (7 days) of crocin (25 and 50 mg/kg) dose-dependently suppressed both phases (3 and 6 h) of carrageenan (1%)-induced paw edema in rats. They also showed that crocin inhibited production of prostaglandin E_2 in rat paw tissues and LPS-challenged RAW 264.7 cells [50]. There are no reports showing the inhibitory effects of crocin and safranal on neutrophil infiltration induced by carrageenan observed in the present study. It has been reported that acute inflammation induced by carrageenan is accompanied by vascular permeability triggered by bradykinin and histamine and subsequent infiltration of neutrophils (0–6 h) followed by monocytes and macrophages (24 h) to the site of inflammation [13]. Boskabady et al. [5] have found that safranal produced anti-inflam-

matory effects in lung inflammation of ovalbumin-sensitized guinea pigs. It also produced anti-inflammatory effects by reducing total white blood cell number in ovalbumin-sensitized guinea pigs [3]. Tamaddonfard et al. [42] reported an anti-inflammatory effect for crocin by reducing paw edema as well as infiltration of neutrophils in paw tissues induced by *ipl* injection of histamine. Crocetin (the aglycone of crocin) protected vascular endothelial-cadherin expression, an adherence protein that has a major role in control of vascular permeability [48]. The results of the present study showed that crocin and safranal attenuated allodynia and hyperalgesia induced by *ipl* injection of carrageenan. Crocin produced antinociceptive effects in hypertonic saline-induced corneal nociception and formalin-induced inflammatory pain [43, 44]. In addition, safranal produced antinociceptive and anti-inflammatory effects in formalin, writhing and hot plate tests in mice [17]. In chronic constriction injury model of neuropathic pain in rats, *ip* injection of safranal, but not crocin, attenuated cold and mechanical allodynia as well as thermal hyperalgesia [1]. In the present study, we showed suppression of cold allodynia and mechanical allodynia and hyperalgesia by crocin and safranal. The differences between our results and the findings of Amin and Hosseinzadeh [1] might be associated with the different kind of animal models of pain. The *ip* injection of crocin at doses of 10 and 20 mg/kg for 15 consecutive days suppressed inflammatory responses in a rat model of arthritis [16]. Taken together, the results of the present study showed anti-inflammatory and antinociceptive effects for saffron constituents, crocin and safranal, in an inflammatory model of pain.

The present results indicated that crocin at doses 50 and 100 mg/kg and safranal at doses of 1 and 2 mg/kg produced anti-inflammatory and analgesic effects resemble to 10 mg/kg of diclofenac did. Diclofenac and other anti-inflammatory drugs such as naproxen and aspirin have abilities to inhibit cyclooxygenase, and were frequently used as reference drugs in studying the anti-inflammatory and analgesic mechanisms of drugs and plant extracts [4, 14, 18, 30, 38, 47]. In FCA model of arthritis in rats, crocin produced better effects than ibuprofen (a NSAID) in reducing paw swelling and serum concentrations of COX-2 and PGE_2 [16]. In addition to the inhibitory effects of crocin and safranal on cyclooxygenase, experimental data have suggested the involvement of reactive oxygen species and inflammatory cytokines for anti-inflammatory and antinociceptive effects of crocin [16, 27]. Recent studies have shown that crocin enhanced morphine-induced analgesia in formalin-induced inflammatory pain and corneal nociception induced by hypertonic saline [43, 44]. However, it has been found that naloxone (an opioid receptors antagonist) did not prevent antinociception induced by crocin [43, 44].

In conclusion, the results of the present study showed that crocin and safranal produced anti-inflammatory and antinociceptive effects in carrageenan model of inflammation and inflammatory pain. The anti-inflammatory and antinociceptive effects of crocin and safranal were the same as those of diclofenac. The inhibition of cyclooxygenase may be involved in the anti-inflammatory and antinociceptive effects of crocin and safranal.

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